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Evolution of Phosphagen Kinase (III). Amino Acid Sequence of Arginine Kinase from the Shrimp *Penaeus japonicus*

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ABSTRACT—The amino acid sequence of arginine kinase (AK) from the shrimp *Penaeus japonicus* has been determined chemically. It consists of 355 amino acid residues, and has a calculated molecular mass of 40,018 Da. The amino acid sequence of *Penaeus* AK showed 91% and 51% identity, respectively, with those of AKs from the lobster *Homarus vulgaris* and the abalone *Nordotis madaka*. It also showed significant homology (39–43%) with vertebrate or invertebrate creatine kinases and annelid glycocyamine kinase, suggesting that these enzymes evolved from a common origin.

INTRODUCTION

Phosphagen kinases (PKs) are the enzymes that catalyze the reversible transfer of the high energy phosphoryl group of ATP to the naturally occurring guanidines, and play a key role to interconnect energy production and utilization in animals [9]. In vertebrates, the only phosphagen kinase is creatine kinase (CK), but in invertebrates, at least five phosphagen kinases, arginine kinase (AK), glycocyamine kinase (GK), taurocyamine kinase (TK), lombricine kinase (LK) and CK, have been identified by partial or complete sequencing [1–3, 15, 18, 20]. Moreover, the presences of hypotaurocyamine kinase (HTK), opheline kinase (OK) and thalassemine kinase (ThalK) are proposed by their enzyme activity [11, 19]. The homologous amino acid sequences of about 15 residues around the putative active site of these enzymes suggest that they have evolved from a common origin [1], and thus provide an excellent model system to elucidate how enzymes developed the recognition site for substrate during evolution.

AK is the phosphagen kinase that is most widely distributed in animals. Very recently, two cDNA-derived amino acid sequences of AKs from the lobster *Homarus vulgaris* [3] and the abalone *Nordotis madaka* [18] have been determined. Here we report the primary structure of AK from the shrimp *Penaeus japonicus*, to be sequenced chemically. A preliminary account of this work has been presented [13].

MATERIALS AND METHODS

AK was isolated from the tail muscle of *Penaeus japonicus* according to our previous method [12].

The protein (50 nmoles) was carboxymethylated and cleaved with CNBr in 70% formic acid. Larger CNBr peptides were digested further with lysyl endopeptidase, chymotrypsin, *S. aureus* V8 protease and pepsin. To obtain overlap peptides, the protein was also digested with lysyl or arginyl endopeptidases. The digestion conditions are the same as described previously [16]. The digested products were purified on a reverse-phase column (Cosmosil $5C_{18}$-300, 2.5×150 mm) with a linear gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 ml/min. Some peptides were purified further by rechromatography. Amino acid analyses and the manual Edman sequencing of the peptides were done with our standard methdos [16]. The N-terminal peptide CN1C1 was digested with acylamino acid releasing enzyme (0.025U, Takara) in 5 mM phosphate buffer (pH 7.2) contianing 1 mM 2-mercaptoethanol at 37°C for 2 hr, before sequencing.

RESULTS AND DISCUSSION

Fig. 1 shows the elution profile of CNBr peptides of *Penaeus* AK on reverse-phase chromatography. Most of the CNBr peptides were separated successfully, and the larger peptides were digested further with several enzymes. Two small CNBr peptides, Gln-Met at positions 233–234 and C-terminal Glu-Lys-Glu-Met, were not recovered. The overlap of CNBr peptides was obtained with the peptides derived from lysyl or arginyl endopeptidase digestions of the whole protein. Amino acid compositions of the CNBr peptides and the whole protein are given in Table 1. The strategy to establish the complete amino acid sequence is shown in Fig. 2. The Gly-Arg bond at position 206–207, Arg-Ala at 279–280, Arg-Gly at 308–309 and Lys-Arg at 327–328 were unusually cleaved with *S. aureus* V8 protease. The sequence is supported by at least two amino acids overlap, and the C-terminal half of 129 residues was also confirmed by the cDNA sequencing [17]. *Penaeus* AK begins with the blocked Val, is composed of total 355 amino acid residues and the molecular mass was calculated to be 40,018 Da.

So far, all the amino acid sequences of phosphagen kinases were determined by their cDNA sequencing, and therefore this work is the first example to be sequenced chemically.

Amino acid sequence of *Penaeus* AK was aligned with *Homarus* AK, *Schistosoma* (trematode) PK domain 1, *Neanthes* (annelid) GK, *Nordotis* (mollusc) AK, sea urchin CK domains 2 and 3 and chicken three CK isoforms (muscle, brain and mitochondrial types), with the algorithm of Feng &

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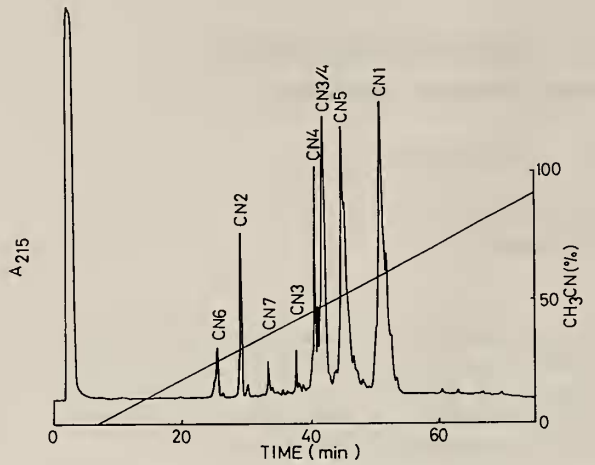

Fig. 1. Elution profile of CNBr peptides of *Penaeus* AK on reverse-phase chromatography. The column (Cosmosil $5C_{18}$-300, 2.5× 150 mm) was eluted with a linear gradient of 0–80% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min.

Doolittle [5], in Fig. 3. It is noted that sea urchin CK has an unusual three-domain structure which may be resulted from a gene triplication [20] and *Schistosoma* PK has a two-domain structure, of which the second domain lacks the C-terminal 50 residues [15]. *Penaeus* AK is aligned with *Homarus* AK without any insertions or deletions. Furthermore, their sequences are characterized by a unique delation at positions 117–118 and an insertion at position 311 in Fig. 3. In the alignment, there are 68 amino acid residues (indicated by asterisks) conserved in all of the phosphagen kinases.

All of the phosphagen kinases can be inactivated partially or completely under the chemical modification with thiol-specific regents [5]. The reactive cysteine, that would be located near or in the center of the putative active site, was identified as Cys-286 (see Fig. 3). Recent site-directed mutagenesis study shows that the active cysteine is necessary to confer conformational changes upon substrate binding, but is not essential for catalysis [6].

The percent identity between the 10 amino acid sequences of phosphagen kinases is shown in Table 2. The sequence of *Penaeus* AK showed 91% and 51% identity, respectively, with those of *Homarus* and *Nordotis* AKs. It also showed significant homology (39–43%) with verebrate or inverebrate CKs and *Neanthes* GK. These sequence homologies would be enough to conclude that CK, GK and AK are derived from a common origin.

A phylogenetic tree was constructed from the sequence alignment in Fig. 3 with the algorithm of Feng & Doolittle [5] (Fig. 4). The same topology was also obtained with the protein parsimonious algorithm using the program **Protpars** in the PHYLIP package ver 3.5c [4]. The tree separated phosphagen kinases into two clusters, a cluster containing vertebrate and invertebrate CKs and invertebrate GK and a cluster containing three AKs and *Schistosoma* PK. The branching pattern clearly shows that CK and GK must have evolved from a common ancestor [18]. The phylogenetic position of *Schistosoma* PK is noted. Our tree placed the PK near the cluster of AKs (Fig. 4). In fact, *Schistosoma* PK has the highest sequence homology (46–52%) with AKs (see Table 2). Moreover, *Schistosoma* PK shares the sequence characteristics with invertebrate AKs: deletions at posi-

Table 1. Amino acid compositions of *Penaeus* AK and its CNBr peptides

	whole	CN1	CN2	CN3	CN3/4	CN4	CN5	CN6	CN7
Asx	37.8(36)	15.4(16)	1.0(1)		8.8(9)	8.6(9)	9.3(9)		1.1(1)
Thr	16.9(19)	5.4(6)	1.0(1)	3.0(3)	3.6(4)	1.0(1)	6.2(7)	1.1(1)	
Ser	15.8(19)	7.1(9)		3.4(4)	5.2(6)	1.9(2)	3.4(4)		
Glx	11.0(11)	11.0(11)	4.1(4)	4.5(4)	11.9(11)	7.5(7)	10.2(9)	3.6(3)	2.3(2)
Pro	10.6(12)	4.8(5)	1.9(2)	0.8(1)	1.9(2)	0.9(1)	2.9(3)		
Gly	31.2(29)	9.5(10)	1.0(1)	3.3(3)	6.2(6)	3.3(3)	9.9(10)	1.1(1)	1.1(1)
Ala	22.6(22)	9.7(10)	1.0(1)		4.0(4)	3.9(4)	6.2(6)	1.1(1)	
Cys	5.0(5)	1.5(2)	0.9(1)		0.8(1)	0.9(1)	1.0(1)		
Val	22.6(22)	9.1(11)		1.2(1)	4.1(4)	3.1(3)	6.0(6)	1.3(1)	
Met	9.7(9)	+ (1)	+ (1)	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)
Ile	15.8(17)	3.8(6)			2.7(4)	2.8(4)	4.5(5)		1.6(2)
Leu	37.0(35)	12.5(13)	1.1(1)	4.3(4)	8.9(9)	4.9(5)	9.7(9)	1.3(1)	1.8(2)
Tyr	11.9(12)	4.4.(5)	1.8(2)	0.5(1)	2.4(3)	1.3(2)	2.0(2)		
Phe	18.9(18)	6.4(7)	1.0(1)	1.1(1)	5.1(5)	3.9(4)	4.2(4)	1.3(1)	
Lys	26.8(29)	12.0(13)	1.0(1)	2.0(2)	6.0(6)	4.0(4)	6.0(6)	1.0(1)	1.0(1)
His	10.1(9)	1.7(2)			2.9(3)	2.7(3)	3.8(4)		
Arg	15.6(17)	2.7(3)			3.7(4)	3.6(4)	9.5(10)		
Trp	+ (2)				+ (2)	+ (2)			
Total	(355)	130	17	25	85	70	96	11	10
Position		1–130	131–147	148–172	148–232	173–232	235–330	331–341	342–351
Yield(%)		57.9	42.3	9.0	35.4	15.6	41.0	34.5	46.7

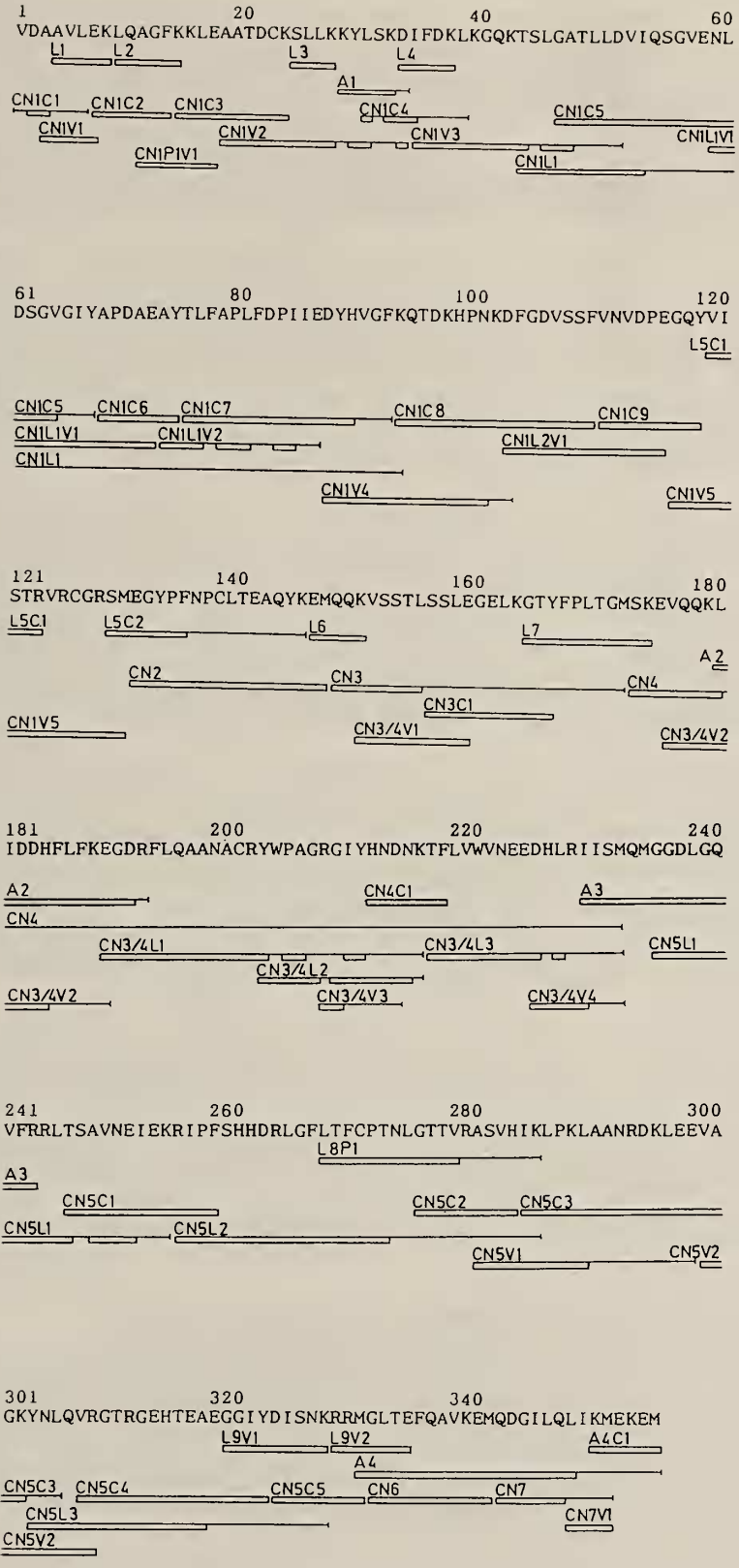

FIG. 2. Summary of data to establish the amino acid sequences of *Penaeus* AK. The sequence was determined by manual Edman degradation (□). Key; CN, CNBr; L, lysyl endopeptidase; C, chymotrypsin; V, *S. aureus* V8 protease; P, pepsin; A, arginyl endopeptidase.

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                                25                          50
AK  shrimp     VDAAVLEKLQ AGFKKLEAATDCKSLLKKYLSKDIFDKLKGQKTSLGATLLDVIQSGVENLD
AK lobster    MADAATIAKLE EGFKKLEAATDCKSLLKKYLSKDIFDSLKAKKTSLGATLLDVIQSGVENLD
AK abalone       MLAMASVE ELWAKLDGAADCKSLLKNNLTKERYEALKDKKTKFGGTLADCIRSGCLNLD
PK Schis.1        MQVESLQ NLQAKIRNDERNHSLTKKYLTDDIVKKYQATKTSLGGTLAQCVNTNAYNPG
CK-M chick   PFSSTHNKHKLKFSAEEEFPDLSKHNNHMAKVLTPELYKRLRDKETPSGFTLDDVIQTGVDNPGH
CK-B chick   PFSNSHNLLKMKYSVDDEYPDLSVHNNHMAKVLTLDLYKKLRDRQTSSGFTLDDVIQTGVDNPGH
CK sea ur2                    YPDLSKHNNHLAHCLTYDIWKSLKDKKTPSGFTLDGCIQTGVMNPGH
CK sea ur3                    YPDFSLHNNWMSKCMTEEIYNKLCNLKTKGGVTLNDCIQTGIDNPGH
CK-Mt chic      TVHEKRKL FPPSADYPDLRKHNNCMAECLTPAIYAKLRDKLTPNGYSLDQCIQTGVDNPGH
GK ma.worm      MFKDYSREKF AKENFPDLSKHNNVMASHLTYELYEKYWDKVTPNGVTLDKCIQTGVDNPGN
                                                  *  *  *      *

                    75                        100                       125
AK  shrimp       SGVGIYAPDAEAYTLFAPLFDPIIEDYHVGFKQTDKHPNKDFGDVSS  FVNVDPEGQYVI
AK lobster       SGVGIYAPDAEAYSLFAPLFDPIIEDYHKGFKQTDKHPAKDFGDVSK  FINVDPEGTFVI
AK abalone       SGVGIYACDPDAYTVFADVLDAVIKEYHKV  PELKHPEPEMGDLDKLNFGDLDPSGEYIV
PK Schis.1       ALLPR SCDLNAYETFRDFFDAVIADYHKVPDGKIQHPKSNFGDLKSLSFTDLNTYGNLVV
CK-M chick   PFI MTVGCVAGDEESYEVFKDLFDPVIQDRHGGYKP TDKHRTDLNHENLKGGDDLDP  KYVL
CK-B chick   PFI MTVGCVAGDEESYEVFKELFDPVIEDRHGGYKP TDEHKTDLNADNLQGGDDLDP  NYVL
CK sea ur2   PHI MTVGMVAGDEESYDVFADIFDPVIDARHGGYPK DAVHVTNINHADLKGGDNLDP  KYVL
CK sea ur3   PYI MTVGLVAGDEECYEVFAPLFDPVISARHGGYAL DAKHPTNLNAAELKGGDDLDP  EFVL
CK-Mt chic   PFI KTVGMVAGDEESYEVFAEIFDPVIKARHNGYDPRTMKHHTDLDASKITHG QFDE  RYVL
GK ma.worm   KFYGKKTGCVFGDEHSYETFKDFFDRVIEEIHH FKPEDVHPATDLDETKLVGG VFDE  KYVK
                        *   *  *    *  *   *

                              150                       175
AK  shrimp   STRVRCGRSMEGYPFNPCLTEAQYKEMQQKVSSTLSSLEGELKGTYFPLTGMSKEVQQKLIDDHF
AK lobster   STRVRCGRSMEGYPFNPCLTEAQYKEMEEKVSSTLSGLEGELKGSYFPLTGMTKEVQQKLIDDHF
AK abalone   STRVRVGRSHDSYGFPPVLTKQERLKMEEDTKAAFEKFSGELAGKYFPLEGMSKEDQKQMTEDHF
PK Schis.1   STRVRLGRTVEGFGFGPTLTKETRIELENKISTALHNLSGEYEGTYYPLTGCQRGQNQTSKRHHF
CK-M chick   SSRVRTGRSIKGYSLPPHCSRGERRAVEKLSVEALNSLEGEFKGRYYPLKAMTEQEQQQLTDDHF
CK-B chick   SSRVRTGRSIRGFCLPPHCSRGERRAIEKLSVEALGSLGGDLKGKYYALRNMTDAEQQQLIDDHF
CK sea ur2   SCRVRTGRSIIGYSLPPHCTVEERAAVETITIGALDKFDGDLQGKYYPLEGMSDETQTQLIDDHF
CK sea ur3   SCRVRTGRCIRGLALPPCCTRAERAEVEKITTEALSTLSGPLKGKYYPLTGMTDEEQEKLIEDHF
CK-Mt chic   SSRVRTGRSIRGLSLPPACSRAERREVENVVVTALAGLKGDLSGKYYSLTNMSERDQQQLIDDHF
GK ma.worm   SCRIRCGRSVRGVCLPPAMSRAERRLVEKVVSNALGGLKEDLAGKYFPLTTMNDKDMEALIEDHF
             * * * **       *                          * *  *                **

               200                       225                       250
AK  shrimp   LF KEGDRFLQAANACRYWPAGRGIYHNDNKTFLVWVNEEDHLRIISMQMGGDLGQVFRRLTSAV
AK lobster   LF KEGDRFLQAANACRYWPAGRGIYHNDNKTFLVWCNEEDHLRIISMQMGGDLGQVYRRLVSAV
AK abalone   LF KDDDRFLRDAGGYNDWCSGRGIFFNTAKNFLVWVNEEDHLRLISMQKGGDLAAVYKRLVVAI
PK Schis.1   LF RNDDNVLRDAGGYIDWPTGRGIFINKQKKFLVWINEEDHIRVISMQKGRDLIAVYKRLADAI
CK-M chick   LFDKPVSPLLLASGMARDWPDARGIWHNDNKTFLVWVNEEDHLRVISMEKGGNMKEVFRRFCVGL
CK-B chick   LFDKPVSPLLLASGMARDWPDARGIWHNDNKTFLVWINEEDHLRVISMQKGGNMKEVFTRFCTGL
CK sea ur2   LFDKPVSPLLTAARMHRDWPQGRGIWHNENKNFLVWVNEEDHIRVISMEKDGNMRAVFKRFCEGL
CK sea ur3   LFDKPVSPLLLCANMARDWPQGRGIWHNDEKNFLVWVNEEDHTRVISMEKSGNMKRVFERFCDGL
CK-Mt chic   LFDKPVSPLLTCAGMARDWPDARGIWHNNDKTFLVWINEEDHTRVISMEKGGNMKRVFERFCRGL
GK ma.worm   LFEKPTGALLTTSGCARDWPDGRGIWHNNGKNFLVWINEEDHIRIISMQKGGDMRAVFSRFGRGL
             **       *          *   ***  *  * **** ***** * ***         *  *

                        275          $          300                       325
AK  shrimp   NEIE    KR IPFSHHDRLGFLTFcPTNLGTTVRASVHIKLPKLAANRDKLEEVAGKYNLQVRG
AK lobster   NDIE    KR VPFSHHDRLGFLTFcPTNLGTTVRASVHIKLPKLAANREKLEEVAAKFSLQVRG
AK abalone   NTMT    ASGLSFAKRDGLGYLTFcPSNLGTALRASVHMKIPNLAASPE FKSFCDNLNIQARG
PK Schis.1   QELS    KS LKFAFNDRLGFITFcPSNLGTTLRASVHAKIPMLASLPN FKEICEKHGIQPRG
CK-M chick   KKIEEIFKKAGHPFMWTEHLGYILTcPSNLGTGLRGGVHVKLPKLSQHPK FEEILHRLRLQKRG
CK-B chick   TQIETLFKSKNYEFMWNPHLGYILTcPSNLGTGLRAGVHIKLPNLGKHEK FGEVLKRLRLQKRG
CK sea ur2   QKFEQMIKKDGKEFMWNKHLGYVLTcPSNLGTGLRAGVHVKLPLLSKYPR FDQILRALRLQKRG
CK sea ur3   KKVEDSIKSKGYQFMWNEHLGYVLTcPSNLGTGLRAGVHVKVPLLSQQKI FDSILDHMRLQKRG
CK-Mt chic   KEVERLIKERGWEFMWNERLGYVLTcPSNLGTGLRAGVHVKLPRLSKDPR FPKILENLRLQKRG
GK ma.worm   TEVERLMKEKGYELMRNERLGYICTcPTNLGTVVRASVHLRLANLEKDKR FDDFLAKLRLGKRG
                              **    ** ****  *  **     *                    **


                                  350                       375
AK  shrimp   TRGEHTEAEGGIYDISNKRRMGLTEFQAVKEMQDGILQLIKMEKEM
AK lobster   TRGEHTEAEGGIYDISNKRRMGLTEFQAVKEMQDGILELIKIEKEM
AK abalone   IHGEHTESVGGVYDLSNKRRLGLTEYQAVEEMRVGVEACLAKEKELAAAKK
PK Schis.1   THGEHTESVGGIYDLSNKRRLGLTELDAVTEMHSGVRALLELEVMLQEYNKGAPEGV
CK-M chick   TGGVDTAAVGAVFDISNADRLGFSEVEQVQMVVDGVKLMVEMEKKLEQNQPIDDMIPAQK
CK-B chick   TGGVDTAAVGGVFDVSNADRLGFSEVELVQMVVDGVKLLIEMEKRLEKGQSIDDLMPAQK
CK sea ur2   TGGVDTASTDGTFDISNLDRLGSSEVQQVQFVVDGVELLVQMEKKLEKGEDIFDILPQQCRPKPP
CK sea ur3   TGGVDTASTDGTFDISNSDRIGFSEVHLVQQLVDGVKLLVNLEKALMKGEDINSLLPEKLREDSS
CK-Mt chic   TGGVDTAAVADVYDISNLDRMGRSEVELVQIVIDGVNYLVDCEKKLEKGQDIKVPPPLPQFGRK
GK ma.worm   TGGESSLAEDSTYDISNLARLGKSERELVQVLVDGVNVLIEADKRLEAGKPIDDLTPRLNSSTGT
               *          * **  * *  *   *     *

                    400
AK  shrimp
AK lobster
AK abalone
PK Schis.1
CK-M chick
```

```
CK-B chick
CK sea ur2  IKPFSYD
CK sea ur3
CK-Mt chic
GK ma.worm  SISATASRHMTL
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FIG. 3. Alignment of the amino acid sequences of 10 phosphagen kinases. This alignment was obtained with the algorithm of Feng & Doolittle [5]. Invariable residues are indicated by asterisks. The reactive cysteine is shown by $. References; CK-M chick (chicken muscle isoform) [10, 14]; CK-B chick (chicken brain isoform) [7]; CK sea ur2 and 3 (domains 2 and 3 of sea urchin) [20]; CK-Mt chic (chicken mitochondrial isoform) [8]; GK ma.worm (*Neanthes*) [18]; AK shrimp (*Penaeus*) (this work); AK abalone (Nordotis) [18]; AK lobster (*Homarus*) [3]; PK Schis. 1 (domain 1 of *Schistosoma*) [15].

TABLE 2. Percent identity between the sequences of phosphagen kinases

	AK lob	AK aba	PK Sch	CK-M	CK-B	CK ur2	CK ur3	CK-Mt	GK wor
Ak shr	91.0	50.9	46.3	42.5	43.0	42.3	41.7	39.8	38.5
AK lob		52.6	45.7	41.8	41.8	41.7	41.7	39.3	38.8
AK aba			51.8	38.3	36.9	43.3	39.8	37.3	37.1
PK Sch				34.4	35.8	36.8	36.2	35.9	34.6
CK-M					80.3	68.2	65.2	67.2	53.2
CK-B						65.8	65.8	66.7	56.2
CK ur2							68.9	65.5	51.1
CK ur3								64.1	51.0
CK-MT									57.4

Abbreviations: shr, *Peanaeus*; lob, *Homarus*; aba, *Nordotis*; Sch, domain 1 of *Schistosoma*; CK-M, chicken muscle isoform; CK-B, chicken brain isoform; ur2 and ur3, domains 2 and 3 of sea urchi; CK-Mt, chicken mitochondrial isoform; wor, *Neanthes*.

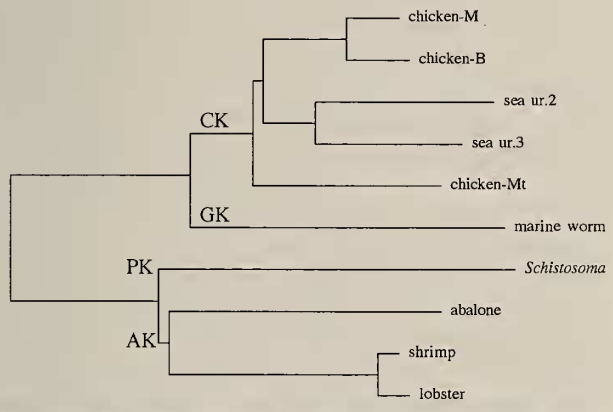

FIG. 4. A phylogenetic tree constructed from 10 sequences of phosphagen kinases aligned in Fig. 2. The tree was obtained with the program of Feng & Doolittle [5].

tions 13, 65–69, 198 and 265–268 in Fig. 3. Stein *et al.* [15] assigned tentatively *Schistosoma* PK as CK, based on the very weak, but reproducible CK activity in the crude extracts. However, since the enzyme activities of CK, GK, AK are strictly specific and those of TK, HTK, LK, OK and ThalK are more or less interspecific [1, 19], it is very likely that *Schistosoma* PK belongs to a member of the latter group. Dumas & Camonis [3] also suggest this possibility, based on the higher % identity between *Schistosoma* PK and lobster AK.

The evolutionary origin of phosphagen kinases is of primary concern. Of the phosphagen kinases, AK is most widely distributed in animals. However the wide distribution does not imply that AK is closer to an ancestral phosphagen kinase. To solve this problem, we are planning to analyze the phosphagen kinases from more primitive animals, such as sea anemones and protozoa.

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